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Mbd3, a component of the NuRD co-repressor complex, is required for development of pluripotent cells

Keisuke Kaji, Jennifer Nichols* and Brian Hendrich[†]

Mbd3 is a core component of the NuRD (Nucleosome Remodeling and Histone Deacetylation) co-repressor complex, and NuRD-mediated silencing has been implicated in cell fate decisions in a number of contexts. Mbd3-deficient embryonic stem (ES) cells made by gene targeting are viable but fail to form a stable NuRD complex, are severely compromised in the ability to differentiate, and show LIF-independent self-renewal. Mbd3 is known to be essential for postimplantation embryogenesis in mice, but the function of Mbd3 *in vivo* has not previously been addressed. Here we show that the inner cell mass (ICM) of Mbd3-deficient blastocysts fails to develop into mature epiblast after implantation. Unlike Mbd3-null ES cells, Mbd3-deficient ICMs grown *ex vivo* fail to expand their Oct4-positive, pluripotent cell population despite producing robust endoderm outgrowths. Additionally, we identify a set of genes showing stage-specific expression in ICM cells during preimplantation development, and show that Mbd3 is required for proper gene expression patterns in pre- and peri-implantation embryos and in ES cells. These results demonstrate the importance of Mbd3/NuRD for the development of pluripotent cells *in vivo* and for their *ex vivo* progression into embryonic stem cells, and highlight the differences between ES cells and the ICM cells from which they are derived.

KEY WORDS: Pluripotency, ES cells, Epiblast, Epigenetics, Mbd3, NuRD

INTRODUCTION

Mammalian embryogenesis begins with a single cell, the zygote, which in the context of the proper maternal environment can give rise to all tissues of the embryo and the placenta. The zygote and cells of early cleavage stage embryos are said to be totipotent, that is, they are capable of giving rise to all embryonic and extra-embryonic tissues (Rossant, 1976). At the morula stage, the embryo begins to compact and by the blastocyst stage the outer cells form an extra-embryonic cell layer called the trophectoderm, while the inside cells form the inner cell mass (ICM) that will eventually give rise to all cells of the foetus and some further extra-embryonic structures. Proper segregation of the ICM and trophectoderm lineages at this stage requires the activity of the transcription factors Cdx2 and Oct4 (*Pou5f1*) (Nichols et al., 1998; Niwa et al., 2005; Strumpf et al., 2005). At 3.5 days post-coitus (dpc), the ICM contains two cell populations, one expressing *Pou5f1* and *Nanog* that is apparently fated to become epiblast, the other expressing *Pou5f1* and *Gata6* and is likely to become primitive endoderm (Chazaud et al., 2006). At late blastocyst stages (around 4.5 dpc) and immediately prior to implantation, the two cell populations become spatially resolved with the primitive endoderm forming a layer of cells facing the blastocyst cavity, while the early epiblast retains the potential to derive all embryonic cell types, a property known as pluripotency. Immediately after implantation, the early epiblast begins to expand and form a columnar epithelium with a proamniotic cavity, a structure that we define here as 'late epiblast'. This transition is accompanied by a dramatic increase in cell proliferation, resulting

in expansion of the pluripotent cell population from ~25 cells at 4.5 dpc to ~660 cells by 6.5 dpc (Snow and Bennett, 1978). In addition to having a shorter cell cycle, the late epiblast also differs from early epiblast in terms of gene expression (Pelton et al., 2002). However, little is known of the differentiation events from 3.5 dpc ICM to early epiblast and late epiblast, owing to the small size and inaccessibility of relevant tissues.

One of the striking differences between the pluripotent cell populations in 3.5 dpc ICMs, early epiblast and late epiblast, is the ability to produce embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981; Smith, 2001). ES cells, which can be maintained indefinitely in culture while maintaining pluripotency, can be derived from cells of the ICM and early epiblast, but not from late epiblast (Brook and Gardner, 1997). ES cells share many properties with ICM/early epiblast cells, and are able to differentiate into all embryonic cell types including the germ line (Bradley et al., 1984). However, ES cells can be derived from only 30% of ICMs at best with conventional methods (Brook and Gardner, 1997), and the molecular mechanisms underlying the derivation of ES cells, and the molecular differences between ES cells and ICM/early epiblast cells, are largely unknown (Zwaka and Thomson, 2005).

The difference between early embryonic pluripotent cells and the lineage-committed cells derived from them, or their transformed derivatives *in vitro* (i.e. ES cells), is, by definition, epigenetic. Therefore, it stands to reason that epigenetic silencing factors will play important roles in this process. Several proteins involved in epigenetic silencing have been shown to be important for early embryonic viability (Bernstein et al., 2003; Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2003; Dodge et al., 2004; Hendrich et al., 2001; O'Carroll et al., 2001; Rayasam et al., 2003), although it is not always clear why the mutant embryos do not survive. ES cells appear to contain an unusual epigenetic profile consisting of both active and silencing histone methylation marks, which tend to resolve into one or the other upon differentiation (Azuara et al., 2006; Bernstein et al., 2006). Many genes which encode developmentally important

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transcription factors are occupied by the Polycomb repressive complexes PRC1 and PRC2 in ES cells, and mouse ES cells deficient for PRC components fail to maintain silencing at several of these loci (Azura et al., 2006; Boyer et al., 2006; Lee et al., 2006). Although the details of epigenetic modifications in pluripotent cells in vivo have been lacking owing to the scarcity of relevant tissue, a recent study has revealed that ICMs have more intense silencing marks than do ES cells, at in least some genes (O'Neill et al., 2006).

The NuRD (Nucleosome Remodeling and Histone Deacetylation) co-repressor complex is an abundant and biochemically well-characterised co-repressor which is implicated in silencing in a number of contexts and in a variety of organisms, including mammals, flies, nematodes and plants (Ahringer, 2000; Bowen et al., 2004). In mammals, NuRD-mediated silencing has been implicated in cell fate decisions during ES cell differentiation (Kaji et al., 2006) and haematopoietic development (Fujita et al., 2004; Hong et al., 2005; Hutchins et al., 2002; Rodriguez et al., 2005; Williams et al., 2004). One of the core components of NuRD is the Mbd3 protein (Wade et al., 1999; Zhang et al., 1999). Mbd3 was originally identified in mice and humans as a protein containing a region with high homology to the methyl-CpG-binding domain (MBD) of MeCP2, but which did not bind methylated DNA (Hendrich and Bird, 1998). Recently, we generated *Mbd3*^{-/-} ES cells by gene targeting and showed that Mbd3 is required for stable formation of the NuRD complex in ES cells (Kaji et al., 2006). *Mbd3*^{-/-} ES cells are viable and maintain expression of genes associated with pluripotent cells such as Oct4 and Nanog, but fail to differentiate or silence expression of Oct4 upon withdrawal of LIF in vitro or in chimeric embryos.

Mice lacking a functional *Mbd3* gene die prior to midgestation (Hendrich et al., 2001), but why these embryos fail to develop, and what role is played by Mbd3/NuRD in early embryos has not been described. In this study, we have characterised the function of Mbd3 in peri-implantation development. We find that Mbd3 is required for ICM cells to develop into late epiblast after implantation, for proliferation of epiblast cells in culture, and for the proper function of extra-embryonic tissues immediately after implantation. We further identify a number of gene expression differences between ICMs from 3.5 dpc embryos, 4.5 dpc embryos and ES cells, many of which show misregulation in mutant embryos prior to the onset of morphological abnormalities. Together, these data demonstrate that Mbd3/NuRD plays a key role in the transition of pluripotent ICM cells towards an epiblast fate during peri-implantation development, and for the acquisition of an ES cell fate.

MATERIALS AND METHODS

Mice

Mbd3^{+/-} mice (Hendrich et al., 2001) were backcrossed to C57Bl/6 mice for more than 10 generations. Hex-GFP mice (Rodriguez et al., 2001) were kindly provided by Joshua Brickman and used to generate *Mbd3*^{-/-} Hex-GFP embryos. Embryonic diapause was induced as described (Buehr and Smith, 2003). To generate GFP-ES cell/*Mbd3* mutant embryo chimeras, Tau-GFP ES cells (Pratt et al., 2000) were aggregated with morulae obtained from *Mbd3* heterozygote intercrosses. After overnight culture, embryos were transferred to the uteri of embryonic day (E) 2.5 pseudopregnant females and collected after 3 days.

Immunofluorescence and RNA in situ hybridisation

Embryos were fixed with 4% paraformaldehyde and permeabilised with 0.25% Triton X-100. After blocking with 1% skimmed milk and 0.05% Tween 20 in PBS, embryos were stained with primary antibodies: anti-Mbd3 (sc-9402, Santa Cruz), anti-Oct4 (sc-8628, sc-5279, Santa Cruz and Becton Dickinson), anti-Nanog (provided by Ian Chambers, Institute for Stem Cell Research, Edinburgh, UK), anti-Gata4 (sc-1237, Santa Cruz), anti- α 7 integrin (provided by Ann Sutherland, University of Virginia,

Charlottesville, VA) (Klaffky et al., 2001), anti-activated caspase-3 (AF-835, R&D Systems), anti-Ki67 (MAB4062, Chemicon), followed by the appropriate secondary antibodies. Nuclei were stained with DAPI (Molecular Probes). Images were captured with a confocal microscope (Leica TSC SP2) and Leica confocal software. Whole-mount RNA in situ hybridisation was performed as described (Rosen and Beddington, 1993). Staining reactions were carried out for 2 days. The in situ probes for *Cdx2* and *Mash2* have been described previously (Buehr et al., 2003; Nichols et al., 1998). Embryos were genotyped by PCR.

Immunosurgery and ICM culture

Immunosurgery was carried out as described (Solter and Knowles, 1975), with collection of trophectoderm lysates for genotype determination by PCR. ICMs were cultured in the presence of 2000 U/ml of LIF and 20% FCS in GMEM on gelatin-coated dishes.

Single-ICM and single-cell PCR

cDNA from single ICMs (30–40 cells) or single cells was amplified as described (Iscove et al., 2002), except for the following modifications. The sequence of the primer used for cDNA amplification is 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGTACT_{30-3'}. SuperRNaseIn (Ambion) and SuperScript III (Invitrogen) were used as RNase inhibitor and reverse transcriptase, respectively. PCR was carried out with ExTaq (Takara). The genotype of individual ICMs was confirmed by PCR with trophectoderm lysates. cDNAs from seven 3.5-dpc ICMs, six 4.5-dpc ICMs, or five single ES cells, respectively, were combined and the amount of cDNA was normalised for Gapdh expression by PCR. See Table 1 for information on gene-specific primers. All PCRs for specific genes were performed with an annealing temperature of 60°C.

RESULTS

Mbd3 is required for proper organisation of ICM-derived tissues upon implantation

We have previously shown that *Mbd3*-deficient ES cells made by gene targeting contained little or no NuRD complex, were severely compromised in their ability to differentiate, and persistently expressed Oct4 in a variety of differentiation conditions including in chimeric embryos (Kaji et al., 2006). To investigate the role played by Mbd3/NuRD in pluripotent cells in vivo, we sought to determine the function of Mbd3 in pre- and peri-implantation embryos. In wild-type embryos, Mbd3 expression was observed in all cells from morulae to the egg cylinder stage at 6.5 dpc, and appeared exclusively nuclear (Fig. 1A). Maternal Mbd3 protein was detected in morulae but not in blastocysts, as judged by immunofluorescence of *Mbd3*^{-/-} embryos produced by intercrossing *Mbd3* heterozygote parents (Fig. 1B). At both 3.5 dpc and 4.5 dpc, *Mbd3*^{-/-} embryos were morphologically indistinguishable from wild-type littermates, showed normal expression of Oct4 at 3.5 dpc and 4.5 dpc (Fig. 2A), and normal segregation of early epiblast (Oct4-Nanog double-positive cells; Fig. 2B) and hypoblast or primitive endoderm (Gata4-expressing cells, some of which also express Oct4; Fig. 2B) at 4.5 dpc. Null embryos were recovered at mendelian frequencies at both 3.5 dpc and 4.5 dpc (Table 2).

By 5.5 dpc, null embryos were no longer identified in the expected ratio and failed to form a cavitated structure, with the Oct4-expressing, presumptive epiblast cells remaining proximally located as in 3.5 and 4.5 dpc embryos (Fig. 2C). *Mbd3*^{-/-} ES-derived cells in chimeric embryos were capable of forming an epiblast-like structure, but failed to extinguish Oct4 expression (Kaji et al., 2006). By contrast, Oct4-expressing cells in *Mbd3*^{-/-} embryos were gradually lost without ever forming a proamniotic cavity (Fig. 2C) while the ectoplacental cone continued to grow during early postimplantation stages, demonstrating that Mbd3 is not required for growth of all cell types. Whereas null embryos

Table 1. PCR primers

Gene name	Primer sequences (5'-3')	
<i>Mbd3</i> + allele	TGTAGCCACCTAGCTCAAGG	CACGCTGGCGACTCTTATTC
<i>Mbd3</i> – allele	TGTAGCCACCTAGCTCAAGG	TCCGCAAACCTCTATTCTG
<i>Mbd3</i> expression	TCCAGGTCTCAGTGCAAGGA	TGACTTCCTGGTGGGCTGCT
<i>Gapdh</i> *	GTGTTCTACCCCAATGTG	GTCATTGAGAGCAATGCCAG
<i>Pou5f1</i>	CCAACGAGAAGAGTATGAGGC	AGAGCAGTGACGGGAACAGAG
<i>Nanog</i>	GTGCATATACTCTCTCTTCCC	AGCTACCCTCAAACCTCTGGT
<i>Pramel4</i> *	GAAAACGCTGCTGCAACATA	GAGCAGTCGGGTGTTCTCTAC
<i>Pramel5</i> *	GCATTCCAGCACTGTCTGAG	TACTGGACCTCCCCAAGAAA
<i>Pramel6</i> *	AGATTCCAGGGCTCATCCTT	ACTTCTTGGGCTGCCTTTT
<i>Pramel7</i> *	AGAGAACCACATGGCTTTG	GGATTGGCTTGGCATAACAT
<i>Ppp2r2c</i>	TGATCGAGGAAAAAGTGCGG	GATACCAACACCCTCCAAG
<i>2410076121Rik</i>	TGAAAGCCAAGGGAAGGACC	CCACCTCTTCCACAAATGCTG
<i>Sohlh2</i>	CCCCCTTCTTCATCATCATC	GCCATCCTTCTCCCTTGTT
<i>Sfrp1</i>	CATGTTGGCTGCTCTGACCT	CAAAACACACCTGGGAATCACT
<i>Aes</i>	CACCTTCAACATTTCCCTGG	GAAGAGGGGAGGGAAAGTTTG
<i>Yaf2</i>	CACTGGTATTTCTGCATCCTC	GGAGTGACACCTCAGTTCTGTG
<i>Dazl</i>	GTCCTTACATGTACCATTCTGTGAC	GACTCCAACAAAACAGCAGACAA
<i>Peg3</i>	CCTGAGGAGGAAGAGAGCAAC	TCAGTCTCAAGGGGTCTGGG
<i>Htra1</i>	TTTGTCCCTTCCCTTAGCCT	GTCAGTGAAGTCCGGGTTCC
<i>Pak1</i>	TGACCAAGCTCTCTGCAAGTG	AGAAACTGTGCTGCTGAGGC
<i>Dppa3</i> *	CTTCCCCAAGAGAAGGGTCC	TGCAGAGACATCTGAATGGC

*Bortvin et al. (Bortvin et al., 2003)

were recovered at sub-mendelian frequencies at 5.5 dpc and 6.5 dpc, by 8.5 dpc the number of null embryos recovered reverted to the expected 25%. This indicates that our failure to isolate some *Mbd3*^{−/−} embryos immediately after implantation is due to their very small size, whereas by 8.5 dpc, null conceptuses have grown a large ectoplacental cone and are easily identified (Table 2 and Fig. 2C).

The failure of *Mbd3*^{−/−} embryos to expand their pluripotent cell population after 4.5 dpc could be due to apoptosis and/or quiescence of these cells. In order to address the possibility of increased cell death in *Mbd3*^{−/−} ICM-derived cells, 5.5 dpc embryos were stained for activated caspase-3, a marker of apoptosis (Krajewska et al., 1997). No Oct4-caspase-3 double-positive cells were found in any *Mbd3*^{−/−} embryos (n=3), and were found only rarely in wild-type

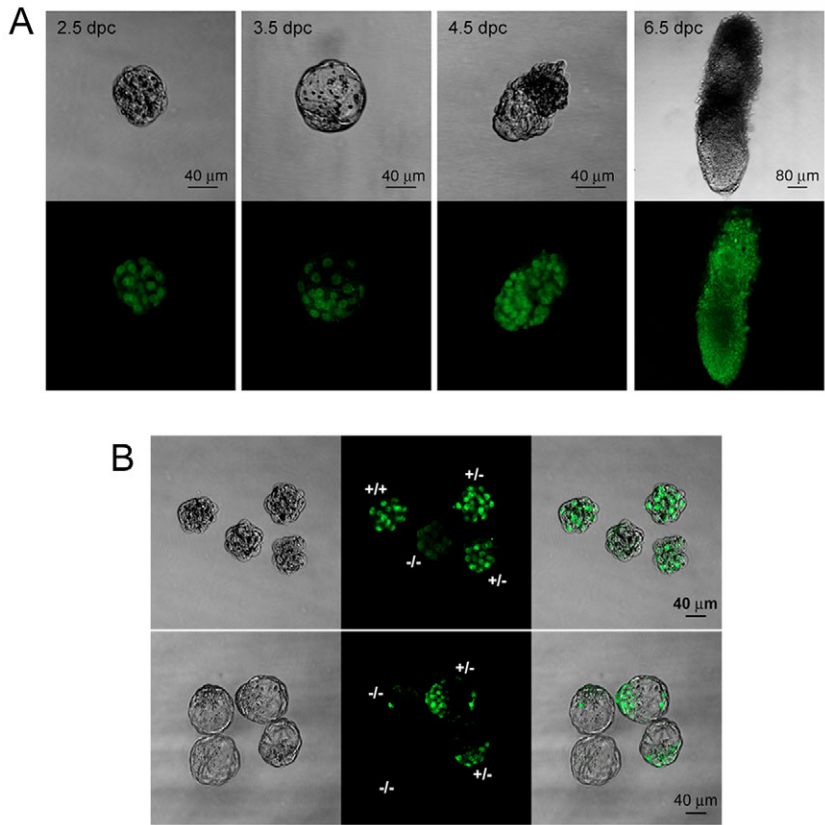


Fig. 1. *Mbd3* expression in pre- and peri-implantation development. (A) Phase contrast microscopy (top panels) and anti-Mbd3 staining (bottom panels) of wild-type embryos at indicated stages. (B) Staining of morulae (top panels) and blastocysts (bottom panels) with anti-Mbd3 antibodies (green; middle panels) produces a strong, nuclear signal in all cells of wild-type and *Mbd3*^{+/−} embryos, but only a faint nuclear signal in the *Mbd3*^{−/−} morulae and no staining in *Mbd3*^{−/−} blastocysts. The genotypes of embryos are indicated in the middle panel.

Table 2. Number and genotype of embryos produced by *Mbd3* heterozygote intercrosses

Age (dpc)	+/+	+/-	-/-	n
3.5	40 (24.0%)	83 (49.7%)	44 (26.3%)	167
4.5	12 (29.3%)	18 (43.9%)	11 (26.8%)	41
5.5	21 (25.9%)	50 (61.7%)	10 (12.3%)	81
6.5	6 (31.6%)	10 (52.6%)	3 (15.8%)	19
8.5	9 (20.5%)	23 (52.3%)	12 (27.3%)	44

embryos (8 of 16 embryos) (see Fig. S1A in the supplementary material). To determine whether loss of *Mbd3* results in quiescence of late epiblast cells, embryos were stained for Ki67 (Mki67), a marker of proliferating cells (Gerdes, 1990). Ki67-expressing cells are detectable in both the extra-embryonic tissues and in the Oct4-expressing cells of both wild-type and *Mbd3*^{-/-} embryos at 5.5 dpc (see Fig. S1B in the supplementary material). Thus, we find no evidence for death or quiescence of ICM descendents in the absence of *Mbd3* at 5.5 dpc.

The finding that *Mbd3*-null ICMs fail to expand their pluripotent cell population was surprising given that *Mbd3*-null ES cells are viable and maintain Oct4 expression even when induced to differentiate in chimeric embryos (Kaji et al., 2006). The epiblast, extra-embryonic ectoderm, and primitive endoderm show a complex interdependence during normal development, such that a defect in one tissue can result in developmental abnormalities in an adjacent tissue (Guzman-Ayala et al., 2004; Murray and Edgar, 2000). Therefore, we next assessed whether other cell types were formed properly in mutant peri-implantation embryos. Double-staining of *Mbd3*^{-/-} 5.5 dpc embryos for Oct4 and $\alpha 7$ integrin, a marker of

extra-embryonic ectoderm and trophectoderm (Klaffky et al., 2001), showed that although the $\alpha 7$ integrin-expressing cells are distinct from and surround the Oct4-expressing cells, mutant embryos do not form an organised extra-embryonic ectoderm structure (Fig. 3A, bottom panels). Similarly, *Mash2* (*Ascl2* – Mouse Genome Informatics) expression, which normally marks the developing ectoplacental cone, was detectable over the entire mutant embryo (Fig. 3B). *Cdx2*, which is normally expressed in trophoblast stem cells and in extra-embryonic ectoderm (Strumpf et al., 2005), was detectable in only a few cells by whole-mount in situ hybridisation in 5.5 dpc *Mbd3*^{-/-} embryos, again consistent with a lack of extra-embryonic ectoderm organisation (Fig. 3B). The fact that *Mbd3*-null embryos could form an ectoplacental cone (Fig. 2C) and were able to implant, indicates that some trophectoderm lineage cells could proliferate and function in the absence of *Mbd3*. Nevertheless, we saw no evidence for expansion of the *Cdx2*-expressing cell population or formation of extra-embryonic ectoderm in mutant embryos after implantation.

Staining embryos for Oct4 and Gata4, an endoderm marker, revealed that although *Mbd3*^{-/-} embryos show normal endoderm specification at 4.5 dpc (Fig. 2B), by 5.5 dpc mutant embryos have failed to form the organised visceral endoderm epithelium seen in wild-type embryos at this stage (Fig. 3C). In addition to the Gata4-positive cells and the Oct4-positive cells, presumably indicating primitive endoderm and primitive ectoderm, respectively, mutant embryos also contained Gata4-Oct4 double-positive cells (*n*=3) (Fig. 3C). Oct4-Gata4 double-positive cells were never observed in wild-type embryos at this stage (*n*=12), but were characteristic of early primitive endoderm cells in the ICMs of 4.5 dpc embryos (e.g. Fig. 2B). This persistence of Oct4-

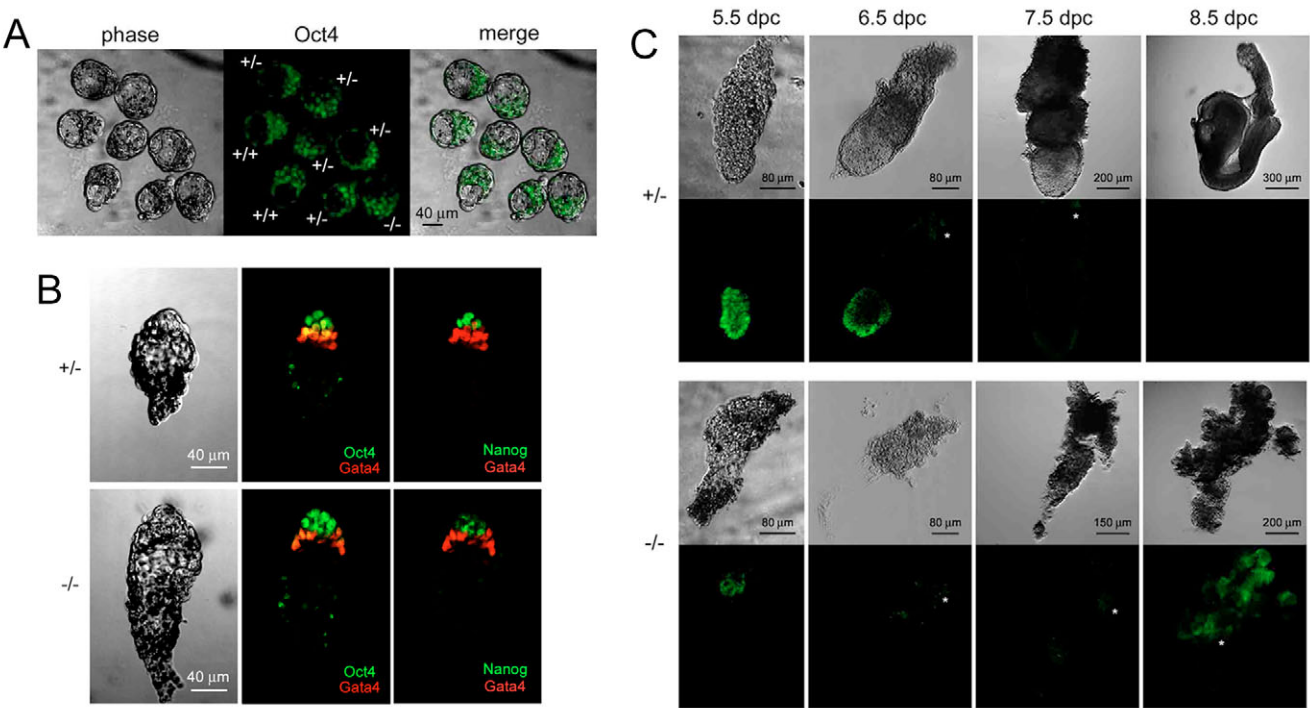


Fig. 2. Abnormal morphology and loss of epiblast after implantation. (A) One litter of embryos at 3.5 dpc was stained for Oct4 expression (green). Genotypes for each embryo are indicated in the middle panel. (B) Embryos at 4.5 dpc were stained with anti-Oct4, anti-Nanog and anti-Gata4 antibodies. Images taken with independent wavelength were coloured in green for Oct4 and Nanog, red for Gata4. Note Gata4-positive primitive endoderm cells maintain Oct4 protein (yellow cells, middle panels) at this stage in both heterozygous and homozygous null embryos. (C) *Mbd3*^{+/+} and *Mbd3*^{-/-} embryos at indicated stages shown in phase contrast (top panels of each set) or stained for Oct4 (green; bottom panels of each set). Asterisks indicate non-specific staining at ectoplacental cone.

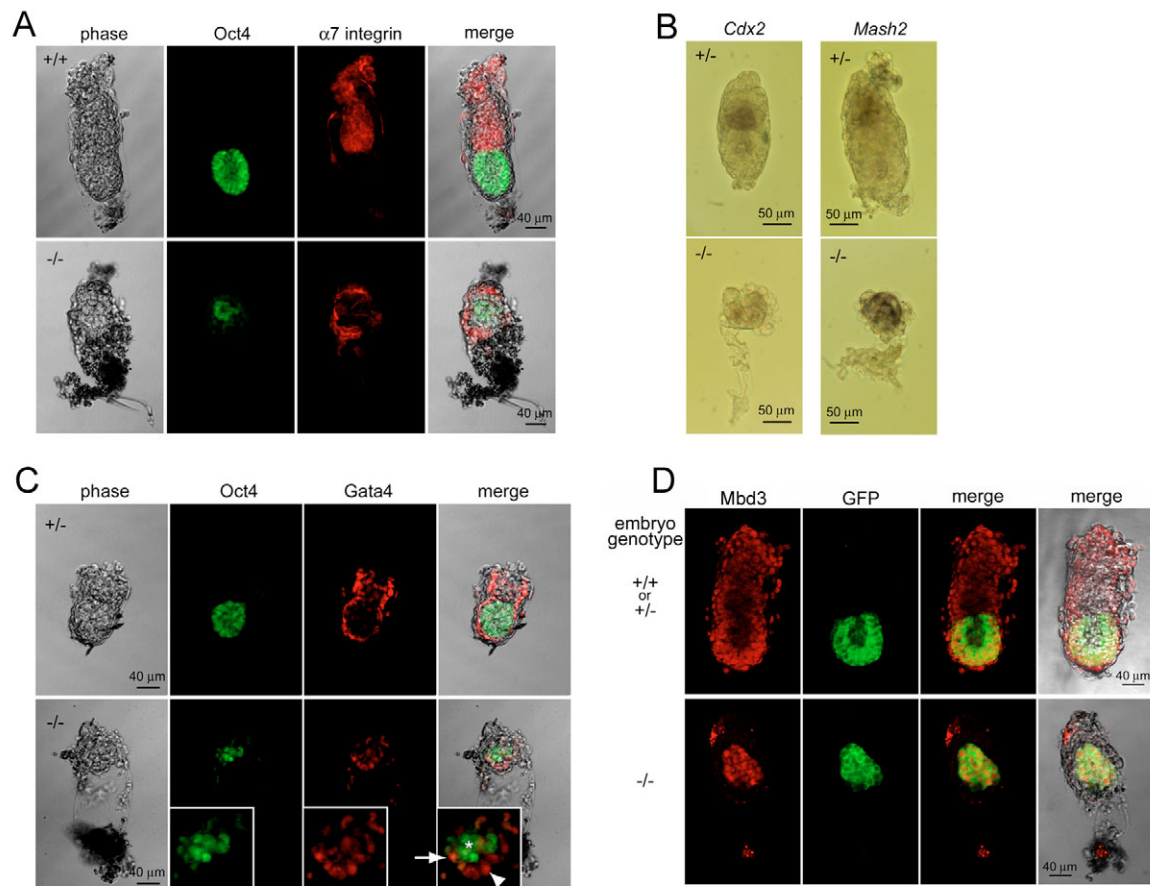


Fig. 3. Mbd3 is required for epiblast maturation and extra-embryonic development. (A) Wild-type (top panels) and homozygous null (bottom panels) embryos at 5.5 dpc were stained for Oct4 (green) and $\alpha 7$ integrin (red). (B) Whole-mount in situ hybridisation of heterozygous (top) and homozygous null (bottom) embryos at 5.5 dpc for *Cdx2* (left-hand panels), and *Mash2* (right-hand panels). (C) Heterozygous (top panels) and homozygous null (bottom panels) embryos at 5.5 dpc were stained for Gata4 (red) and Oct4 (green). A close-up of the homozygous null embryo is shown in the inset at the bottom of each panel to illustrate co-expression of Oct4 and Gata4 in a subset of cells (arrow), in addition to Oct4 or Gata4 single-positive cells (asterisk and arrowhead, respectively). (D) Chimeric embryos made with wild-type ES cells expressing GFP (green) and embryos obtained from *Mbd3* heterozygote intercrosses. The genotype of the embryo used for each chimera was determined by *Mbd3* staining (red) in GFP-negative (i.e. host-derived) cells.

Gata4 double-positive cells in 5.5 dpc *Mbd3*^{-/-} embryos, as well as the lack of robust expression of *Hex* (*Hhex* – Mouse Genome Informatics), a marker of distal visceral endoderm in 5.5 dpc embryos (Rodriguez et al., 2001) (see Fig. S2 in the supplementary material), indicates that primitive endoderm formation is also abnormal in these embryos.

To discern whether *Mbd3* is indeed required for normal function of extra-embryonic tissues, wild-type, GFP-expressing ES cells were aggregated with *Mbd3*^{-/-} morulae and allowed to develop for several days in utero. In morula aggregation experiments with ES cells, the extra-embryonic tissues are formed by the host embryo, whereas the ES cells contribute primarily to the epiblast (Tam and Rossant, 2003). *Mbd3*^{-/-} embryo/ES cell chimeras contained a significant number of GFP-expressing, ES-derived cells at 5.5 dpc; however, they formed neither a cavitated epiblast nor an organised extra-embryonic ectoderm (Fig. 3D; *n*=3). Thus, a wild-type, ES cell-derived epiblast was not able to rescue the *Mbd3*^{-/-} embryonic phenotype at 5.5 dpc (Fig. 3D) or 6.5 dpc (data not shown). We therefore conclude that *Mbd3* is necessary for normal development of both the embryonic and extra-embryonic tissues in pre-implantation embryos.

Mbd3 is required for epiblast expansion in vivo and ex vivo

Given the different developmental capacity of *Mbd3*^{-/-} ES cells and ICM cells in embryos, we sought to characterise more fully the ICM phenotype of *Mbd3*^{-/-} embryos independently of the extra-embryonic phenotype. In order to determine whether the loss of Oct4-expressing cells seen in postimplantation *Mbd3*^{-/-} embryos is due to an autonomous defect, or to some developmental or environmental cue, the preimplantation period was extended by subjecting blastocysts to diapause (Buehr and Smith, 2003). In this context, epiblast cells and primitive endoderm cells are spatially distinct, with the former expressing both Oct4 and *Nanog* while the latter express Oct4 and Gata4 (Fig. 4A). As in non-delayed 5.5 dpc embryos, *Mbd3*^{-/-} blastocysts subjected to diapause for 2 days (i.e. at 5 days after fertilisation) generally contained few, if any, Oct4/*Nanog*-expressing epiblast cells, whereas all embryos contained a Gata4-expressing primitive endoderm layer that was comparable in size to that seen in wild-type or heterozygous littermates (Fig. 4A). Thus, we conclude that the loss of epiblast cells in *Mbd3*-null embryos occurs independently of the process of implantation.

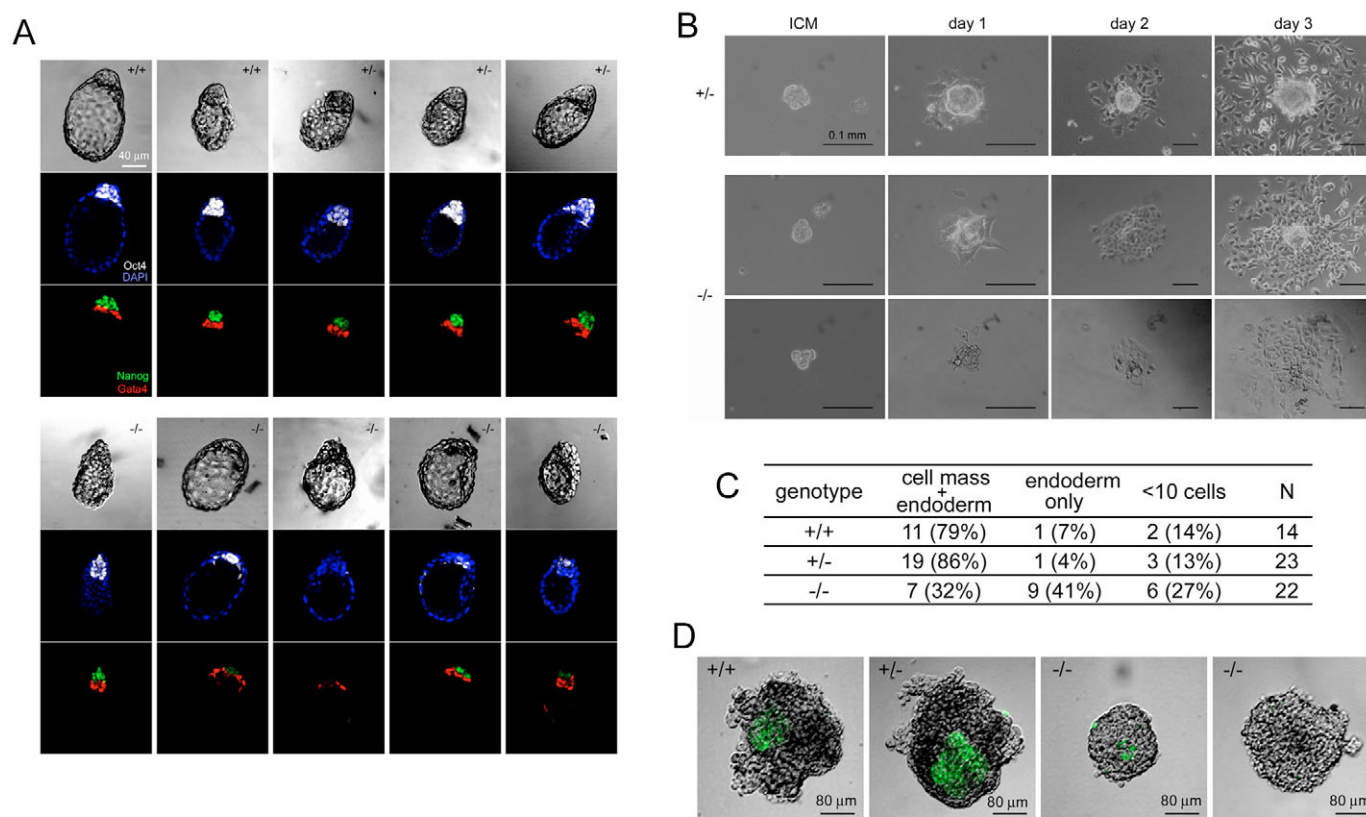


Fig. 4. ICMs lacking *Mbd3* fail to expand pluripotent cells in vivo and in vitro. (A) Embryos in which implantation was delayed by diapause were collected after 3 days of diapause (See Materials and methods) and stained for Oct4 (white), Nanog (green) and Gata4 (red). Nuclei were stained with DAPI (blue). Genotype of embryos is indicated in the phase contrast images. (B) ICMs from *Mbd3*^{+/+} (top panels) or *Mbd3*^{-/-} (middle and bottom panels) 3.5 dpc embryos were isolated using immunosurgery and allowed to attach and outgrow in the presence of LIF for 3 days. Pictures were taken every 24 hours. (C) Number and morphology of ICM outgrowths after 3 days in culture. Any outgrowths with a cell mass in addition to monolayer endoderm were scored as having 'cell mass+endoderm' irrespective of the size of the cell mass. Outgrowths with adherent cells only were classed as 'endoderm only'. Outgrowths containing fewer than 10 cells after 3 days were classed as '<10 cells'. The number of outgrowths of each genotype is indicated (N). (D) Cell masses isolated from ICM outgrowths cultured for 5 days were stained for Oct4 (green). Genotypes are indicated in each panel.

To exclude any possible influence of the trophectoderm on the growth of mutant epiblasts, we next investigated the growth potential of isolated *Mbd3*^{-/-} ICMs ex vivo. ICMs from 3.5 dpc blastocysts were isolated away from the surrounding trophectoderm by immunosurgery and cultured in the presence of LIF. In most cases, the outgrowths from *Mbd3*^{-/-} ICMs were distinguishable from those of wild-type or *Mbd3*^{+/+} ICMs from the first or second day of culture, producing a smaller cell mass and tighter cell junctions between outgrowing endoderm cells (Fig. 4B). After 3 days in culture, only 32% of *Mbd3*^{-/-} outgrowths contained a recognisable cell mass as compared with 79% or 86% of wild-type or heterozygous outgrowths, respectively (Fig. 4C). *Mbd3*^{-/-} cell mass outgrowths were invariably smaller than those produced by wild-type or heterozygous ICMs, and contained few if any Oct4-positive cells (Fig. 4B,D). By contrast, the endoderm produced by *Mbd3*^{-/-} outgrowths expressed Gata4 and Sparc, a marker of differentiated parietal endoderm (Holland et al., 1987) (data not shown). Thus, we conclude that although dispensable for primitive endoderm growth, *Mbd3* is required for the expansion of Oct4-expressing cells during ICM culture that is essential for ES cell derivation (Buehr and Smith, 2003).

Misregulation of gene expression in *Mbd3*^{-/-} ICMs

Mbd3 is a crucial component of the NuRD co-repressor complex (Kaji et al., 2006). Therefore, the failure of *Mbd3*^{-/-} ICMs to expand their pluripotent cell population is likely to be due to aberrant gene expression patterns in the ICMs of *Mbd3*-null preimplantation embryos. We reasoned that gene expression changes resulting in the observed embryonic phenotypes, rather than occurring as a secondary consequence of them, should be present in *Mbd3*-null ICMs after the loss of maternally-derived *Mbd3* protein, but prior to the onset of morphological abnormalities, i.e. in 3.5 dpc and 4.5 dpc ICMs. To identify such genes, we monitored gene expression in wild-type and *Mbd3*^{-/-} 3.5 dpc and 4.5 dpc ICMs by RT-PCR (Fig. 5). To make cDNA from individual ICMs, we used a single-cell cDNA amplification method that includes multiple amplification steps (see Materials and methods for details). In order to control for possible threshold effects and/or amplification bias, cDNA was also made from single ES cells and compared with ES cell cDNA prepared using a conventional method. As shown in Fig. 5, the amplified cDNA produced identical results to those obtained using conventional cDNA for a number of control genes (*Mbd3*, *Gapdh*, *Oct4*, *Nanog*, *Pramel4*, *Pramel5*). It is noteworthy that the reported decrease in *Nanog* expression in 4.5 dpc ICMs (Chambers et al.,

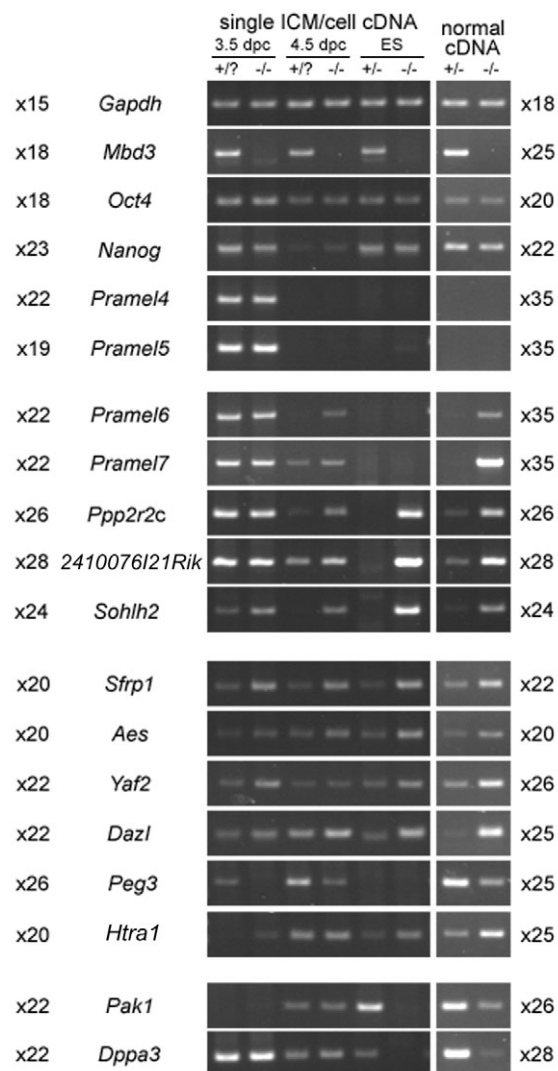


Fig. 5. Gene expression in 3.5 dpc ICMs, 4.5 dpc ICMs and ES cells. Amplified cDNA from individual ICMs and single ES cells were mixed according to the genotype and gene expression was assayed by semi-quantitative PCR. cDNA made with a conventional method from 1 μ g total RNA of ES cells was used to assess the quality of amplified cDNA. The number of PCR cycles for each gene is shown alongside (left for amplified cDNA, right for conventional ES cell cDNA). '(+/?)' indicates embryos of either (+/+) or (+/-) genotype.

2003; Saba-El-Leil et al., 2003) is detectable in our ICM cDNAs, and indicates some degree of normal ICM development between 3.5 and 4.5 dpc in the absence of Mbd3 (Fig. 5).

By monitoring expression of genes found to be misexpressed in *Mbd3*^{-/-} ES cells (K.K. and B.H., unpublished), we identified 11 genes showing altered expression patterns in *Mbd3*^{-/-} ICMs at 3.5 and/or 4.5 dpc (Fig. 5). Expression of three of these genes (*Pramel6*, *Pramel7*, *Peg3*) could not be detected in amplified ES cell cDNA, probably owing to low expression levels, although expression was detected in ICM samples and in conventionally prepared ES cell cDNA. The expression patterns of genes found to be misexpressed in *Mbd3*^{-/-} ICMs fall broadly into two categories. *Pramel6*, *Pramel7*, *Ppp2r2c*, *2410076I21Rik* and *Sohlh2* were all expressed in wild-type 3.5 dpc ICMs, but expression was either decreasing or extinguished in 4.5 dpc ICMs (Fig. 5). *Mbd3*^{-/-} ICMs failed to silence appropriately any of these genes at 4.5 dpc, as did *Mbd3*^{-/-} ES cells. The remaining six

genes either showed no expression changes over this period (*Sfrp1*, *Aes*, *Yaf2*), or showed an increase in expression between 3.5 and 4.5 dpc (*Dazl*, *Peg3*, *Htra1*) in wild-type ICMs (Fig. 5). Each of these genes showed increased expression in *Mbd3*^{-/-} ICMs and ES cells, except *Peg3*, which showed decreased expression. It is notable that misexpression of *Sohlh2*, *Sfrp1*, *Aes*, *Yaf2*, *Dazl* and *Htra1* could already be detected by 3.5 dpc in mutant ICMs. It is not clear which of these genes, if any, are direct targets of NuRD-mediated silencing in ICMs; however, this failure of efficient silencing might indicate that the observed developmental failure of pluripotent cells in *Mbd3*-null ICMs derives from inappropriate gene expression initiating at or before 3.5 dpc (Fig. 6).

DISCUSSION

Epigenetic silencing is important for early development and ES cell derivation

ES cells can be efficiently derived from the ICMs of 3.5 dpc embryos, 4.5 dpc ICMs and from delayed blastocysts (Brook and Gardner, 1997). *Mbd3*^{-/-} embryos can be recovered at each of these stages that contain Oct4-Nanog double-positive cells; however, this presumptive epiblast precursor population fails to expand in vivo or to outgrow in culture. This finding is surprising given the fact that Mbd3 is not required for viability or self-renewal of ES cells (Kaji et al., 2006). These data, along with our identification of differential gene expression between 3.5 dpc ICMs, 4.5 dpc ICMs and ES cells, demonstrate that although each of these cell types are pluripotent, they differ from each other in terms of gene expression and requirements for expansion; ES cells, which in practice are most often derived from 3.5 dpc ICMs, are most similar to 4.5 dpc ICMs. In addition, we show that Mbd3/NuRD is necessary for proper gene expression in 3.5 dpc and 4.5 dpc ICMs, and is important for development of the ICM-derived cell population in peri-implantation embryos.

Several epigenetic silencing proteins have been reported to be important for early murine development (Bernstein et al., 2003; Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2003; Dodge et al., 2004; Hendrich et al., 2001; O'Carroll et al., 2001; Rayasam et al., 2003), and often a requirement in ES cells is inferred from the failure of blastocysts to outgrow in culture. Here, we show that Mbd3, a component of the NuRD co-repressor complex, is required for the initial step of ES cell derivation, namely expansion of the early epiblast cell population, despite being dispensable for ES cell self-renewal. This requirement in early embryonic development and for the growth of ICM cells in vitro, but not for ES cell maintenance, has been reported for only one other epigenetic silencing protein, namely Dicer, a protein important in RNAi-mediated gene silencing (Bernstein et al., 2003; Kanellopoulou et al., 2005; Murchison et al., 2005). Recently, O'Neill et al. identified differences in histone acetylation patterns between ICMs and ES cells, possibly indicating epigenetic changes important for ES cell derivation from ICMs (O'Neill et al., 2006). Together, these data indicate that epigenetic silencing plays an important role in the developmental and gene expression changes that occur during the development of pluripotent cells in vivo and for ES cell derivation.

Mbd3/NuRD is required for programmed maturation of pluripotent ICM cells and development of extra-embryonic tissues

Maternal Mbd3 protein is lost in embryos by 3.5 dpc, at which point altered gene expression patterns are detectable in ICM cells, followed by the appearance of morphological abnormalities within 48 hours. *Mbd3*^{-/-} embryos at 5.5 dpc lack a mature late epiblast, proper spatial organisation and distinct extra-embryonic ectoderm and visceral

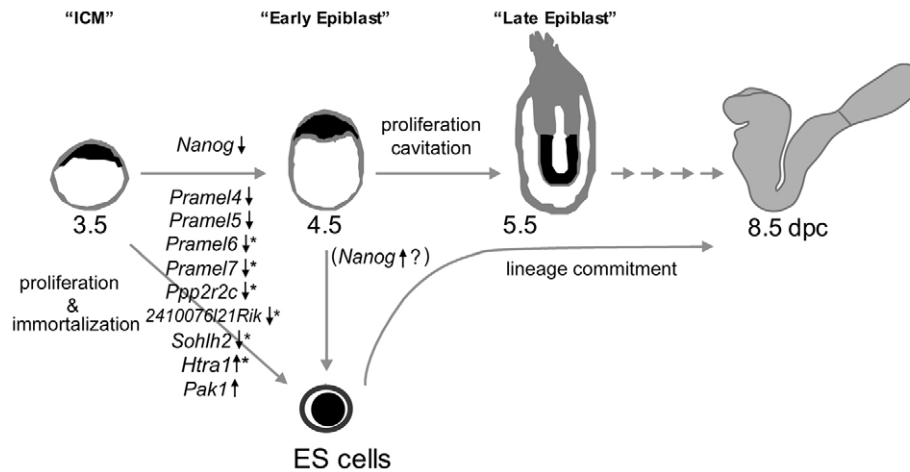


Fig. 6. Transition of pluripotent cells and function of *Mbd3*. Pluripotency (indicated as black shading in cells and embryos) is passed on from the 3.5 dpc ICM to the early epiblast of 4.5 dpc embryos, and then to the late epiblast of 5.5 dpc embryos. During this transition, the expression levels of the listed genes change as shown. Loss of *Mbd3* results in misregulation of genes with asterisks at 3.5 dpc and/or 4.5 dpc, followed by failure of epiblast proliferation and proamniotic cavity formation at 5.5 dpc. Whereas *Nanog* downregulation occurs between 3.5 and 4.5 dpc, it is not clear whether expression levels increase during ES cell derivation, or whether ES cells are derived from rare *Nanog* high-expressing cells at 4.5 dpc. *Mbd3*/NuRD is required for proliferation of ICM cells in vivo and for isolated ICMs to give rise to a proliferating epiblast in the presence of LIF in culture. *Mbd3* is also required for lineage commitment in ES cells (Kaji et al., 2006).

endoderm structures, although they do contain cells expressing markers for each of these lineages. Epiblast cells in 5.5 dpc *Mbd3*^{-/-} embryos display characteristics of 3.5 dpc ICM cells, such as a lack of expansive growth or cavitation, proximal localisation and the presence of Oct4-Gata4 double-positive cells. *Mbd3*-null embryos lose Oct4-expressing cells by 7.5 dpc, while the ectoplacental cone continues to grow. Given that we could find no evidence that the Oct4-expressing cells either die by apoptosis or undergo cell cycle arrest, it may be that they fail to make the transition from ICM cells to become late epiblast, and instead all gradually lose Oct4 expression while activating Gata4 expression and become primitive endoderm.

Although the epiblast, extra-embryonic ectoderm and primitive endoderm interact with each other during normal development at implantation, we feel it is unlikely that the failure of epiblast development seen in *Mbd3*^{-/-} embryos in vivo can be explained solely by failure of one of the extra-embryonic tissues. *Erk2* (Mapk1 – Mouse Genome Informatics) mutant embryos can form a cavitated epiblast without forming either an ectoplacental cone or extra-embryonic ectoderm (Saba-El-Leil et al., 2003), whereas embryos lacking *Elf5*, a transcription factor belong to the Ets superfamily, can develop a cavitated epiblast while lacking an extra-embryonic ectoderm (Donnison et al., 2005), indicating that extra-embryonic ectoderm is not strictly required for epiblast growth in vivo. We cannot rule out the possibility that the defect we see in *Mbd3*^{-/-} ICM cells is actually caused by some defect in very early trophectoderm cells. However, we feel this is very unlikely given the fact that ES cell derivation is possible from *Cdx2*-null embryos, which lack any detectable trophectoderm functions (Meissner and Jaenisch, 2006), demonstrating that early failure of trophectoderm lineages does not preclude ICM outgrowth in vitro. Although *Cdx2*-null blastocysts appear to have trophectoderm-like cells, these cells express *Nanog* and thus may not actually be trophectoderm (Strumpf et al., 2005).

It is also unlikely that the *Mbd3*-null embryonic phenotype is exclusively due to an epiblast defect, as wild-type ES cells were unable to rescue the mutant phenotype in chimeric embryos, and embryos lacking an epiblast due to mutation in *Foxd3*, a member of the forkhead family of transcriptional regulators, are able to form

extra-embryonic ectoderm (Hanna et al., 2002). In addition, whereas most *Mbd3*^{-/-} ICMs were able to give rise to adherent primitive endoderm cells expressing Gata4, even these cells showed a more compact growth morphology than did wild-type primitive endoderm outgrowths, indicating some defect in proliferation and/or adhesion consistent with the disorganised visceral endoderm seen in mutant embryos at 5.5 dpc. Further analysis of *Mbd3*-deficient extra-embryonic tissues and of *Mbd3*-null trophectoderm stem cells and endoderm stem cells (Kunath et al., 2005; Tanaka et al., 1998) will be important for defining the tissue-specific and general requirements for *Mbd3*/NuRD during early postimplantation development.

Transcriptional misregulation in *Mbd3*^{-/-} ICMs

Although it is becoming clear that in 3.5 dpc ICMs there may be distinct cell populations fated to be either epiblast or primitive endoderm with distinct gene expression patterns (Chazaud et al., 2006; Kurimoto et al., 2006), gene expression changes that accompany the maturation of epiblast cells upon implantation remain largely uncharacterised. We identified 11 genes that show altered expression patterns in 3.5 and/or 4.5 dpc ICMs in the absence of *Mbd3*/NuRD. *Sohlh2*, *Sfrp1*, *Aes* and *Dazl* show increased expression immediately after loss of maternally-derived *Mbd3* protein at 3.5 dpc, and misregulation persists in 4.5 dpc ICMs and in ES cells, making these genes good candidates to be predominantly and directly regulated by *Mbd3*/NuRD. *Sfrp1* is a secreted antagonist of the Wnt signaling pathway (Kawano and Kypta, 2003); *Aes* is a naturally occurring dominant-negative molecule alleviating transcriptional repression by TLE/GRG proteins, which bind and repress various transcription factors including Tcf proteins, the nuclear mediators of canonical Wnt signaling (Brantjes et al., 2001). There are several Wnt pathway molecules expressed in preimplantation embryos that can function in both canonical and non-canonical Wnt pathways (Kemp et al., 2005). Therefore, it will be interesting to determine whether misregulation of *Sfrp1* and *Aes*, alone or in combination, can affect the normal development of pluripotent cells.

The two other genes showing early misexpression, *Dazl*, an RNA-binding translational regulator, and *Sohlh2*, a basic helix-loop-helix (bHLH) protein, are both exclusively expressed in germ cells

(Ballow et al., 2006; Ruggiu et al., 1997). By contrast, expression of another germ cell-specific gene, *Dppa3* (also called *Stella* or *Pgc7*), is not disturbed in *Mbd3*^{-/-} ICMs despite the fact that it is strongly downregulated in *Mbd3*^{-/-} ES cells (Fig. 5) (Kaji et al., 2006). Thus, it is unlikely that misregulation of *Dazl* and *Sohlh2* in *Mbd3*^{-/-} ICMs reflects some commitment towards the germ cell lineage; however, it remains possible that their overexpression in *Mbd3*^{-/-} ICMs and ES cells interferes with normal development and/or lineage commitment of pluripotent cells.

The *Pramel* (PRAME-like) genes encode proteins similar to the human preferentially expressed antigen in melanoma, or PRAME, protein (Bortvin et al., 2003). PRAME is an antigen expressed in a wide variety of human tumours (van Baren et al., 1998) and was recently shown to act as a dominant repressor of retinoic acid receptor signalling and to inhibit retinoic acid-induced differentiation of mouse embryonal carcinoma cells (Epping et al., 2005). Although the function of the *Pramel* proteins is not known, their preimplantation-specific expression pattern in mouse (Fig. 5), and the existence of families of *Pramel* genes in the genomes of other mammals, *Xenopus tropicalis* and *Gallus gallus*, might indicate that they play some conserved role in early vertebrate development (Birtle et al., 2005) (B.H., unpublished observations).

Molecular and transcriptional differences between ICM cells and ES cells

ES cells are derived from ICMs, inheriting their pluripotency and acquiring the ability for indefinite self-renewal (Brook and Gardner, 1997). ES cell derivation remains an inefficient process, the efficiency of which varies between different inbred mouse strains and is currently not possible in other rodent species (Buehr and Smith, 2003; Robertson, 1987). Despite the growing popularity of ES cell research, surprisingly little is known about the molecular differences between ICM/epiblast cells and ES cells. In addition to identifying *Mbd3*-dependent gene expression patterns in the ICMs of preimplantation stage embryos and ES cells, we also found seven genes (*Pramel4*, *Pramel5*, *Pramel6*, *Pramel7*, *Ppp2c2r*, *2410076121Rik* and *Sohlh2*) downregulated and 2 genes (*Htra1* and *Pak1*) upregulated between 3.5 dpc and 4.5 dpc in wild-type ICMs (Fig. 5). The expression of most of these genes in wild-type ES cells is more similar to that in 4.5 dpc ICMs than in 3.5 dpc ICMs. These molecular data support the assertion that the origin of ES cells is early epiblast rather than 3.5 dpc ICM (Brook and Gardner, 1997). This indicates that during ES cell derivation, pluripotent cells in 3.5 dpc ICMs must first mature in culture to a 4.5 dpc-like state before undergoing the necessary epigenetic changes that enable them to become ES cells (Fig. 6). ICMs at 4.5 dpc, which include the early epiblast, also show some gene expression differences when compared with ES cells. *Nanog* is robustly expressed in ES cells, but its mRNA levels are decreasing in 4.5 dpc ICMs (Chambers et al., 2003), and *Pak1* expression is also lower than in ES cells (Fig. 5). By contrast, expression of *Pramel7*, *2410076121Rik* and *Htra1* is higher in 4.5 dpc ICMs than in ES cells. Two genes (*Pak1* and *Dppa3*) showed no significant misregulation in *Mbd3*^{-/-} ICMs, although these genes are misregulated in *Mbd3*^{-/-} ES cells (Fig. 5) (Kaji et al., 2006), indicating a difference in control of epigenetic silencing between ICM cells and ES cells.

The effect of loss of *Mbd3* is different in 4.5 dpc ICMs than in ES cells. *Pak1* and *Dppa3* expression is misregulated in *Mbd3*^{-/-} ES cells, but not in *Mbd3*^{-/-} 4.5 dpc ICMs. Furthermore, whereas *Mbd3*^{-/-} ICM cells fail to initiate the rapid proliferation characteristic of late epiblast and do not maintain expression of Oct4, *Mbd3*^{-/-} ES cells show only a modest growth defect and robustly express Oct4 even when induced to differentiate (Kaji et al., 2006). While we

cannot exclude the possibility that some of the differences observed here come from the presence of primitive endoderm cells in 4.5 dpc ICMs, these data further illuminate the differences between ICM cells and ES cells. Although the molecular events that occur during ES cell derivation are largely unknown, here we show that *Mbd3* is necessary for ICM cells to gain ES cell-like properties of increased proliferation and an appropriate gene expression profile necessary for both in vivo development and ES cell derivation.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/6/1123/DC1>

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